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14. ABSTRACT
During our third year of research, we have successfully generated high quality mRNA sequencing and mRNA array data. Linear modeling followed by gene set enrichment analysis reveal connections in cancer gene expression throughout the airway. Specifically, we found that cancer genes in the distal airway are enriched in genes up in cancer in the mainstem bronchus. Similarly, cancer genes enriched in the bronchus and main carina show moderate enrichment in the nose. Our studies reveals spatially connected gene expression patterns in the airway of patients with lung cancer and demonstrated that the adjacent airway field of cancerization is comprised of markers that can identify lung cancer among smokers as well as gradient and localized site-dependent expression patterns that recapitulate NSCLC profiles. Our findings on the adjacent field of cancerization provide additional insights into the biology of NSCLC and the development of molecular tools for the detection of the malignancy. We have collected epithelial samples throughout the respiratory from smokers with and without lung cancer using common SOPs across all 4 participating institutions. We also used a unique approach to profile cell populations from the normal airway, premalignant lesions and tumors and were able to validate these genes. We have established proteomics methods required to validate our candidates in bronchial specimens during year 4 of the award.

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Lung Cancer

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Specific Aim 1: To increase our understanding of the molecular basis of the pathogenesis of lung cancer in the “field cancerization” that develops in current and former smokers.

Investigating molecular signature of lung cancer development in bronchial specimens by reverse phase protein array (RPPA)

We investigated the expression of a group of selected proteins in bronchial brushings and biopsies from low risk and high risk individuals without lung cancer and lung cancer patients by reverse phase protein array (RPPA). Risk level of individuals without lung cancer was calculated based on Bach model criteria (age, gender, smoking history and asbestos exposure) (Bach et al. JNCI 2003). The goal is to identify proteins and pathways altered by risk factors in the field of cancerization. The hypothesis is that a subset of functionally significant molecular alterations in the airway epithelium represents early steps in tumorigenesis, and its measure in individuals at risk for lung cancer development may allow us to derive new insights into tumorigenesis and a tool for risk assessment that will provide the basis of patient selection for surveillance programs.

Methods

We conducted two experiments referred as experiment 1 and experiment 2 using two independent sets of airway specimens. The first experiment was performed using pairs of bronchial brushings and biopsy specimens from 15 patients, 5 per group (low risk, high risk, and cancer group). Brushings and biopsies were collected from the same individual at the same time. The second experiment was performed using biopsies only, each group consisting of 10 biopsies. All experiments were conducted under IRB approved protocol.

Specimen collection procedure: Bronchial brushings were collected from patients under conscious sedation. The brushings were immediately dipped into 1.5 ml saline taken in a labeled eppendorf tube. The tube was kept on ice to minimize protease action. Care was taken to keep the brush specimen free from blood. Brushings in the saline was vigorously agitated by vortexing for about 10 seconds with highest speed. It was then spun 1500g for10 minutes in a microcentrifuge with the brush inside the tube. Supernatant was removed carefully leaving as little saline as possible keeping the brush inside the tube. The pellet was stored in freezer at -80^{o}C temperature. Patients undergoing autofluorescence bronchoscopy for clinical suspicion of lung cancer agreed to provide bronchial biopsy specimens at predetermined normal sites (with normal fluorescence ratio). Biopsy specimens collected for research were snap frozen and stored in -80^{o}C freezer. RPPA was performed according to the published protocol (Byers, LA et al. Cancer Discovery, 2012).

Statistical Methods: ANOVA or two sample t test is applied on a marker-by-marker base to test whether there is difference between the two sources within three sample groups. ANOVA and Tukey's HSD test is applied on a marker-by-marker base to test whether there are difference among the three risk groups and with comparisons contribute to the difference. Because of the multiple testing involved in this approach, the individual ANOVA p-values are not particularly meaningful. However, when we look across the entire set of tests, the distribution of the p-values (under the null hypothesis that no RPPAs provide useful information) should be uniform. If, on the other hand, some RPPAs provide useful information about predicting the response, we would expect an overabundance of small p-values. We can capture this situation by modeling the distribution of the p-values with a Beta-uniform Mixture (BUM). To identify significantly differentially expressed RPPAs, we choose a cutoff for the single test p-values by controlling the false discovery rate (FDR), which is defined as the percentage of RPPAs called significant that are expected

Results

Hierarchical cluster analysis of the results from first experiment was performed with bronchial brushings and bronchial biopsy specimens separately. Applying linear regression model we checked whether the RPPA data show different expression patterns for the three groups. Heatmaps were generated using RPPA data from brushings and biopsy specimens comparing three groups at FDR level of 0.55 and 0.3 respectively (Figure 1). Four out of five brushings of the cancer group was clustered together (Figure 1. Left panel) and all five biopsies from cancer group were grouped together Figure 1. Right panel). Among the classifiers of both type of specimens, ATM, ERCC1 and Rab25 demonstrated overexpression in specimens from cancer patients.

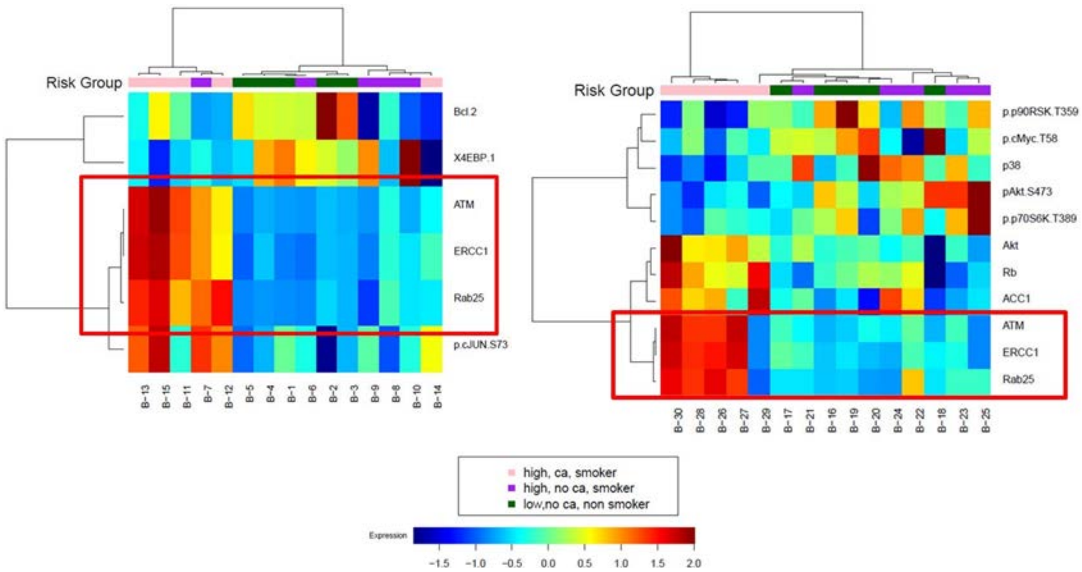

Figure 1. Left panel. Heatmap of classifiers from bronchial brushings specimens comparing three groups at the FDR level of 0.55. Right Panel. Heatmap of classifiers from bronchial biopsy specimens comparing three groups at the FDR level of 0.3.

The second experiment was performed with an independent set of bronchial biopsy specimens. Bronchial brushings were not used in this experiment. Comparison of three groups resulted two main clusters as sown in figure 2. Left cluster includes all control biopsies, 2 out of 10 high risk biopsies and 4 out of 10

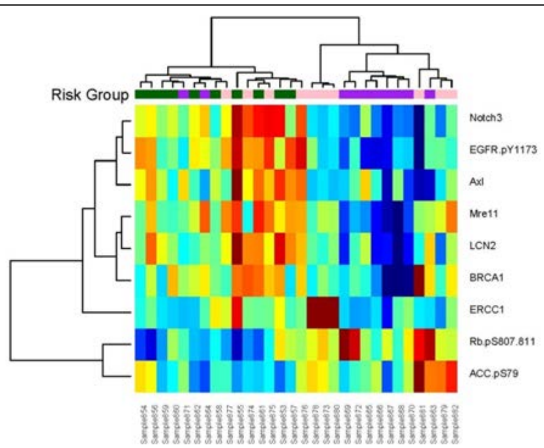

Figure 2. Heatmap of classifiers from bronchial biopsy specimens comparing three groups at the FDR level of 0.25.

biopsies from cancer group. Right cluster is consisting of rest of the high risk and cancer biopsies. Upregulation of Notch3, EGFRpY1173, Axl, Mre11, LCN2 and BRCA1 in almost all samples in the left cluster and downregulation in the right cluster are nicely demonstrated. Unlike experiment 1, ERCC1 was upregulated only in 3 out of 10 biopsies of the cancer group. Rb.pS807.811 was overexpressed in the right cluster and underexpressed in the left cluster. ACC.pS79 was overexpressed in 3 biopsies of the cancer group.

Combining low risk and high risk individuals without cancer and comparing with patients with cancer of the second experiment resulted clusters that are not based on cancer status. Overall two clusters are noticeable mainly based on the expression of ATMpS1981 and ERCC1 in 3 out of 10 patients of the cancer group. Modest overexpression of these proteins was also observed in three low risk, one high risk and one cancer specimen. TSC2.pT1462 is overexpressed in patients without cancer (Figure 3. Left pane).

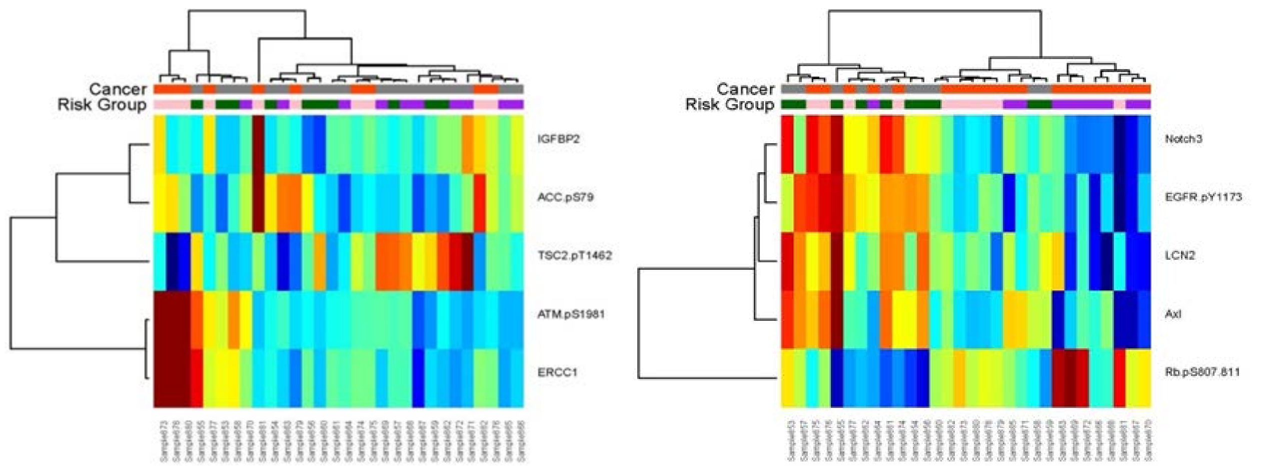

Figure 3. Heatmap of classifiers from bronchial biopsy specimens comparing cancer versus no cancer with top 5 markers, IGFBP2, ACCpS79, TSC2pT1462, ATMpS1981 and ERCC1 (left panel) and Heatmap of classifiers from bronchial biopsy specimen comparing non-smoker versus smoker with top 5 markers, Notch3, EGFR.pY1173, LCN2, AXL and Rb.pS807.811 (right panel).

Next, smokers without cancer and cancer patients who are also smokers were combined and compared with low risk, non-smoking individuals, all from the second experiment. Two main clusters are identified in the heatmap of non-smoker versus smoker (Figure 3. Right panel). Two low risk clustered with high risk and cancer groups (right cluster). Notch 3, EGFRpY1173, LCN2, and Axl are downregulated in this cluster and overexpressed in 8 out of 10 nonsmokers (left cluster). Five out of 20 smokers (high risk and cancer groups) clustered with nonsmokers (low risk groups) where these proteins are overexpressed. Unlike above 4 markers Rb.pS807.811 was overexpression in 5 of the 20 smokers (right cluster) and downregulated in the other cluster.

The data from the second experiment was analyzed by separating the biopsies based on the subtypes of lung cancer found in the third group, i.e. adenocarcinoma (Figure 4. Left panel) and squamous cell (Figure 4. Right panel) carcinoma. There is indication of stronger classification of risk groups for squamous cell carcinoma subtype compared to adenocarcinoma.

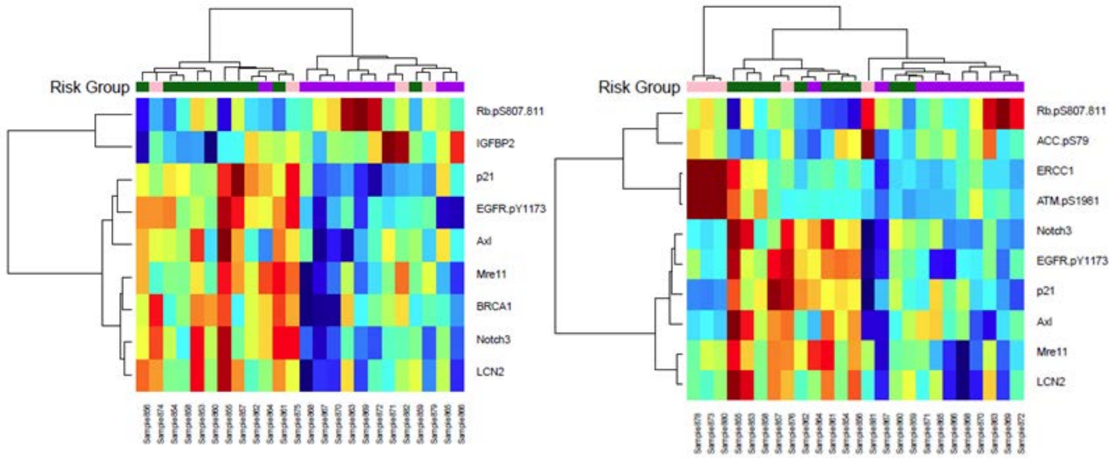

Figure 4. Heatmap of classifiers from bronchial biopsy specimens comparing low risk, high risk and adenocarcinoma only (left panel) and squamous cell carcinoma only (right panel) at the FDR levels of 0.55 and 0.15 respectively.

Conclusions

- Bronchoscopy specimens like brushings and biopsy, can be used interchangeably for RPPA profiling.
- Differential protein expression was demonstrated to be the same in bronchial brushings and bronchial biopsy from the same patient by RPPA.
- Molecular alterations in the bronchial biopsy specimens of at risk individuals that includes epithelium and submucosa could provide a signature for risk assessment.
- Among the differentially expressed candidate proteins ATM and ERCC1 are highly discriminatory among the groups suggesting DNA damage and repair activation in the high risk group.

Future directions

In order to validate the results of the two experiments in a larger dataset we are currently acquiring biopsy specimens for the three groups described above. We will perform RPPA experiments with an expanded list of antibodies. In addition to the 140 proteins that include 125 proteins of the first experiment, we will investigate expression of metabolic enzymes because of possible metabolic reprograming of the airway epithelium, the field of cancerization, of at risk individuals which is indicated in the findings from another project in the laboratory. Expression of selected high ranking candidate proteins will be validated in specimens from collaborators at BU, UCLA and MDA.

Sample acquisition

Table . Seventeen patients collected for RNA extraction at VUMC. Those 10 highlighted were shipped to BU for sequencing analysis.

DOD pt. case	MRN	Patient ID	LCB ID	Sample Type	Fixative	Sample ID	Storage Temperature in Centigrade	Location of Brushes
Vanderbilt 11	33567728	8841	2012-4-1-800-1	Normal Tissue	RNA Later	534048	-80	Tumor is central - LUL
				Normal Tissue		534049	-80	B1 closest to tumor
				Tumor Tissue	RNA Later	534050	-80	B2 same bronchus as B1 - peripheral
				Tumor Tissue		534051	-80	B3 and B4 - different airway -
				Brushes B1-B4	Qiazol	534052-55	-80	
Vanderbilt 12	34227843	8836	2012-4-1-802-1	Normal Tissue	RNA Later	534293	4	Tumor is peripheral (s/p chemorad) - RUL
				Normal Tissue		534294	-80	B3 closest to tumor
				Tumor Tissue	RNA Later	534295	4	B2 same airway as B3 - more proximal
				Tumor Tissue		534296	-80	B1 different airway - proximal
				Brushes B1-B3	Qiazol	534299-301	-80	
								scant tumor left on specimen after chemorad
Vanderbilt 13	34026005	8844	2012-4-1-803-1	Normal Tissue	RNA Later	534326	4	Tumor is central - LUL
				Normal Tissue		534327	-80	B1 closest to tumor
				Tumor Tissue	RNA Later	534329	4	B2 same airway as B1 - distal
				Tumor Tissue		534328	-80	B3 different airway
				Brushes B1-B3	Qiazol	534330-32	-80	
Vanderbilt 14	31374218	9002	2012-5-1-811-1	Normal Tissue	RNA Later	535568	4	Tumor is peripheral - RLL
				Normal Tissue		535569	-80	B4 closest to tumor
				Tumor Tissue	RNA Later	535570	4	B1 and B2 same airway as B4 - more proximal
				Tumor Tissue		535571	-80	B3 different airway - proximal
				Brushes B1-B4	Qiazol	535572-75	-80	
Vanderbilt 15	33737560	9006	2012-5-1-814-1	Normal Tissue	RNA Later	535692	4	Tumor is central - LUL
				Normal Tissue		535690	-80	B1 closest to tumor
				Tumor Tissue	RNA Later	535693	4	B2 and B4 same airway as B1 - distal
				Tumor Tissue		535691	-80	B3 different airway
				Brushes B1-B4	Qiazol	535694-97	-80	
Vanderbilt 16	8993586	9047	2012-6-1-821-1	Normal Tissue	RNA Later	536361	4	Tumor is Central - LUL
				Normal Tissue		536360	-80	B1 is closest to tumor
				Tumor Tissue	RNA Later	536363	4	B2 on a different airway than B1 distal.
				Tumor Tissue		536362	-80	B3 on an opposite airway distal.
				Brushes B1-B3	Qiazol	536364-66	-80	
Vanderbilt 17	15991904	9078	2012-6-1-824-1	Normal Tissue	RNA Later	536782	4	Tumor is peripheral - LLL
				Normal Tissue		536781	-80	B1 is distal to tumor
				Tumor Tissue	RNA Later	536784	4	B2 is closest to tumor on same airway as B1.
				Tumor Tissue		536783	-80	B3 is on a different airway distal.
				Brushes B1-B3	Qiazol	536785-87	-80	
Vanderbilt 18	32690034	9138	2012-7-1-828-1	Normal Tissue	RNA Later	537331	4	Tumor is peripheral - LUL
				Normal Tissue		537330	-80	B1 is closest to tumor.
				Tumor Tissue	RNA Later	537329	4	B2 on same airway as B1 distal.
				Tumor Tissue		537328	-80	B3 is on a different airway distal.
				Brushes B1-B3	Qiazol	537332-34	-80	
Vanderbilt 19	34594325	9258	2012-8-1-840-1	Normal Tissue	RNA Later	538648	4	Tumor is perripheral - LLL
				Normal Tissue		538649	-80	B2 is closest to tumor on the same airway as B1.
				Tumor Tissue	RNA Later	538646	4	B3 and B4 are distal to tumor on different airway.
				Tumor Tissue		538647	-80	
				Brushes B1-B4	Qiazol	538650-653	-80	
Vanderbilt 20	3467849	9341	2012-9-1-842-1	Normal Tissue	RNA Later	539306	4	Tumor is peripheral - RUL
				Normal Tissue		539307	-80	B1 is distal to tumor.
				Tumor Tissue	RNA Later	539304	4	B2 is distal on same airway as B1.
				Tumor Tissue		539305	-80	B3 is on a different airway distal.
				Brushes B1-B4	Qiazol	539308-311	-80	B4 is proximal to the tumor on same airwaiy as B2 and B1.
Vanderbilt 21	23268162	9401	2012-9-1-848-1	Normal Tissue	RNA Later	539677	4	Tumor is peripheral - RUL
				Normal Tissue		539678	-80	B1 is proximal to tumor on same airway as B2.
				Tumor Tissue	RNA Later	539675	4	B2 is distal to tumor.
				Tumor Tissue		539676	-80	B3 is distal to tumor on different airway.
				Brushes B1-B3	Qiazol	539679-681	-80	
Vanderbilt 22	19271741	9519	2012-11-1-862-1	Normal Tissue	RNA Later	541376	4	Tumor is peripheral -RUL
				Normal Tissue		541377	-80	B2 is proximal to tumor on same airway as B1.
				Tumor Tissue	RNA Later	541378	4	B1 is distal to tumor.
				Tumor Tissue		541379	-80	B3 is distal to tumor on different airway.
				Brushes B1-B3	Qiazol	541380-382	-80	
Vanderbilt 23	35129154	9659	2013-1-1-876-1	Normal Tissue	RNA Later	542448	4	Tumor is peripheral - RUL
				Normal Tissue		542449	-80	B1 is closest to tumor.
				Tumor Tissue	RNA Later	542451	4	B2 is distal to tumor on same airway as B1.
				Tumor Tissue		542450	-80	B3 is distal to tumor on different airway.
				Brushes B1-B4	Qiazol	542452-454	-80	
Vanderbilt 24	35655885	9979	2013-4-1-896-1	Normal Tissue	RNA Later	544671	4	Tumor is peripheral - RUL
				Normal Tissue		544672	-80	B1 is proximal to tumor, about .5cm from tumor.
				Tumor Tissue	RNA Later	544673	4	B2 is distal to tumor, about 2.5 cm from tumor. B2 and B1 on same airway.
				Tumor Tissue		544674	-80	B3 is distal, about 3cm from tumor and is on different airway.
				Brushes B1-B3	Qiazol	544675-677	-80	
Vanderbilt 25	29961091	10477	2013-9-1-917-1	Tumor Tissue		546981	-80	
				Tumor Tissue	RNA Later	546982	4	
				Normal Tissue		546983	-80	Tumor is central - Left pnemonectomy
				Normal Tissue	RNA Later	546984	4	B1 is distal to tumor
				Brushes B1-B3	Qiazol	546985-987	-80	B2 is closest to tumor. B2 and B3 are in the same airway.

Specific Aim 2: Evaluate the role of airway epithelium tumor-initiating stem/progenitor cells in current and former smokers.

Summary of Research Findings:

A. Assessment of the molecular profiles of tumor-initiating stem/progenitor cells from normal airway epithelium, premalignant lesions and cancer.

I collaboration with the Dubinett laboratory, the Liebler laboratory is analyzing proteomic characteristics of Snail-driven malignant conversion in human bronchial epithelial cells (HBEC). Snail overexpression drives anchorage-independent growth (AIG). The goal of these analyses is to determine the requirements for P53, KRAS, or both P53/KRAS for full Snail-driven malignant conversion in vivo. Dr. Tonya Walser of the Dubinett laboratory has generated Snail over-expressing HBEC and HBEC-mutant NRH cell lines. Snail expression was confirmed by western blot and cells were confirmed as mycoplasma-negative. Genotyping confirmed P53 and KRAS mutation status and AIG assays were completed. Dr. Walser provided the following cell line pellets to the Liebler laboratory: HBEC2-Snail, HBEC2-vector control, HBEC11-Snail, HBEC11-vector control, H3mutP53/KRAS#12-Snail and H3mutP53/KRAS#12-vector control. Three independent replicate samples of each cell type are being analyzed on a standardized shotgun proteomics platform, in which cell proteins are digested with trypsin and fractionated by basic reverse phase chromatography. Fifteen concatenated fractions from each sample are then analyzed by reverse phase liquid chromatography-tandem mass spectrometry on a Thermo Orbitrap Elite instrument. These analyses are in progress and will be completed by approximately October 31st.

Specific Aim 3: Test airway-based mRNA and microRNA biomarkers of diagnosing lung cancer in current and former smokers at high risk for lung cancer in minimally invasive sites.

Due to the use of both next generation RNA sequencing and comprehensive microarray profiling and due to this ongoing study's unique design we anticipate that expression profiles in the NSCLC molecular field of injury will harbor molecules, both novel and established, that may exhibit potential for use as airway biomarkers that can be developed and tested for lung cancer detection using minimally invasive sites in Specific Aim 3 of this award.

As mentioned in Specific Aims 1 and 2 above, we have identified profiles in the field of injury/cancerization that are also enriched in the nasal compartment of patients with lung cancer relative to patients with benign disease. While microarray (MD Anderson and BU) and RNA sequencing (BU) data analyses are being completed, all four sites/institutions are continuing to collect nasal and airway samples from patients with lung cancer and BU, Vanderbilt and UCLA are continuing to collect nasal and airway samples from patients without lung cancer or benign disease. These new cases will serve as sets to develop and validate classifiers, that are based on profiles from Aim 1 as mentioned above, that can be analyzed readily in the clinic (e.g. by qRT-PCR) in minimally invasive sites (e.g. nasal compartment) in smokers with indeterminate nodules. In addition, to the fourteen lung cancer cases that have already been profiled in Specific Aim 1, as mentioned above, additional 43 lung cancer cases comprised of large airways, airways adjacent to the nearby lung tumor and nasal epithelia, have been collected at MD Anderson Cancer Center. These additional cases will be utilized for development of the classifier in Year 4 of the grant period.

Reportable Outcomes

Abstracts:

- Maki Y, Fujimoto J, Yoo SY, Gower A, Shen L, Garcia MM, Kabbout M, Chow CW, Hong WK, Kalhor N, Wang J, Moran C, Spira A, Coombes KR, Wistuba II, Kadara H. Transcriptomic architecture of the airway field cancerization in early-stage non-small cell lung cancer. 104th Annual American Association for Cancer Research (AACR) meeting, April 6 - April 10 2013, Washington, D.C. Abstract # 2367.
- Ooi AT, Gower AC, Zhang K, Vick J, Hong LS, Fishbein M, Nagao B, Wallace WD, Elashoff DA, Dubinett S, Lenburg M, Spira A, Gomperts BN. Gene expression alterations in premalignant lesions from the airways of patients with lung squamous cell carcinomas. Platform presentation and travel award. AACR Washington DC, April 2013.

Manuscripts:

- Ooi AT, Gower AC, Zhang KX, Vick JL, Hong L, Nagao B, Wallace WD, Elashoff DA, Walser TC, Dubinett SM, Pellegrini M, Lenburg ME, Spira A, Gomperts BN. Profiling premalignant lesions in lung squamous cell carcinomas identifies mechanisms involved in stepwise carcinogenesis. Cancer Research, submitted and under revision.
- Kadara H, Fujimoto J, Yoo SY, Maki Y, Gower AC, Kabbout M, Garcia MM, Chow CW, Chu Z, Mendoza G, Shen L, Kalhor N, Hong WK, Moran C, Wang J, Spira A, Coombes KR, Wistuba II. Transcriptomic architecture of the adjacent airway field cancerization in non-small cell lung cancer. Journal of the National Cancer Institute. Submitted and Under Revision.
- Perdomo C, Campbell JD, Gerrein J, Tellez C, Garrison CB,, Walser TC, Drizik E, Si H, Gower AC, Vick J, Anderlind C, Jackson JR, Mankus C, Schembri F, O'Hara C, Gomperts BN, Dubinett SM, Hayden P, Belinsky SA, Lenburg ME, Spira A. miR-4423 is a Primate-Specific Regulator of Airway Epithelial Cell Differentiation and Lung Carcinogenesis. Proc Nat Acad Sci USA, accepted.